RESEARCH ARTICLE

Effect of Different Concentrations of Amphiregulin/Neurgulin/ Tumour Necrocis Factor α on *in vitro* Maturation of Ovine Oocytes

Ramesh H. Somashetty,[1*] Nandi Sumanta,[2] Girish K. Venkatesh[1]

Abstract

The objective of the present study was to examine the effect of different concentrations of ovarian peptides Amphiregulin (AREG) or Neuregulin-I (NRG-I) or Tumour necrosis factor (TNF-α) on *in vitro* maturation of ovine oocytes collected from different sizes of ovarian follicles. Oocytes isolated from different ovarian follicles (large antral follicles (LAFs>4mm), medium antral follicles (MAFs<1 mm) and small antral follicles (SAFs,1-4mm)] were cultured with medium supplemented with different concentrations viz. 0 (control), 5, 10, 25, 50, 100, and 150 ng of either AREG or NRG-I or TNF-α in a carbon dioxide incubator for 24 h. Maturation rate assessment was done based on the degree of expansion (cumulus expansion score) and extrusion of the first polar body. The maturation rate of oocytes was significantly ($p < 0.05$) higher in groups exposed to 50 ng, 100 ng and150 ng of AREG in oocytes isolated from LAFs; 100 ng and150 ng of AREG in oocytes isolated from both MAFs and SAFs. The maturation rate of oocytes isolated from LAFs, MAFs and SAFS was significantly ($p < 0.05$) higher in the groups exposed to 25 ng, 50 ng, 100 ng and 150 ng of NRG-I; 50 ng,100 ng and 150ng of NRG-I, respectively. The maturation rates were significantly ($P<0.05$) higher in the oocytes cultured with 25 ng of TNF-α in oocytes isolated from both LAFs and MAFs and 5 ng and 10 ng of TNF-α in oocytes isolated from SAFs. The results indicated growth factors AREG/NRG-I/TNF-α improved the oocytes maturation *in-vitro.*

Keywords: Cumulus oocytes complexes, Maturation, Follicle, Ovine, LAFs, MAFs, SAFs.

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Introduction

Ovarian folliculogenesis is a dynamic process characterized by proliferation and differentiation of the somatic cell components mainly regulated by gonadotropins. In addition to this, a variety of locally active peptide/protein / growth factors are also known to be produced by ovarian follicles. These factors can either interact by autocrine or paracrine or juxtacrine or intracrine fashion. Despite being recognized as soluble physiologically active compounds, they are generated as integral membrane precursors with a single membrane-spanning domain (Yardenand Slwikowski, 2001).

In-vitro maturation (IVM) of oocytes is a technique required for laboratory production of embryos. Several ovarian peptides, including AREG and NRG-1, have been found to increase cumulus expansion and oocyte maturation through paracrine factors released by oocytes *in-vitro* (Richani *et al*., 2013 and Noma *et al*., 2011). Removal of the immature oocytes from different size follicles decreased surrounding somatic cells (granulosa and theca cells) effects on oocyte maturation. Mimicking the ovarian follicle system *in vitro* by employing Epidermal growth factor (EGF) like peptides on oocytes was a challenge for making IVM successful (Gall *et al*.2004). Furthermore, Conti *et al*. (2012) reported that the influences of EGF-like growth factors such as Amphiregulin (AREG) and Neuregulin-I(NRG-I) on oocyte nuclear and cytoplasmic maturation were indirect and dependent on cumulus cells.

On the other hand, Sugimura *et al*. (2014) suggested that AREG cooperated with Bone Morphogenetic Protein-15 (BMP-15) to improve the oocyte developmental competence. According to Soares *et al*. (2015), AREG stimulated nuclear maturation faster, and deletion of the Amphiregulin (AREG) / Epidermal growth factor receptor (EGFR) resulted in the inhibition of oocyte maturation. Thus, the objective of the present study was to examine the effect of AREG or NRG-I or TNF-α at different concentrations (0, 5,10, 25,50,100,150 ng/

[1]Veterinary College, KVAFSU, Bangalore Campus, Hebbal, Bangalore, Karnataka, India

[2]ICAR-National Institute of Animal Nutrition and Physiology, Adugodi, Bangalore, Karnataka, India

Corresponding Author: Ramesh H. Somashetty, Veterinary College, KVAFSU, Bangalore Campus, Hebbal, Bangalore, Karnataka, India, e-mail: ramamabhi@gmail.com

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mL) on *in vitro* maturation of ovine oocytes collected from different sizes of ovarian follicles.

Materials and Methods

Chemicals used in the present study were procured from Sigma chemicals, MO, USA.

Collection and Evaluation of Oocytes from Surface Antral Follicles

The ovine (sheep) ovaries collected from a civil slaughterhouse, Bangalore, Karnataka, India, were brought to the laboratory in warm (32 to 33°C) normal saline supplemented with gentamicin (50 µg/mL). The visible ovarian follicles were classified into: small antral follicles (SAFs, <1 mm), medium antral follicles (MAFs,1–4mm), and large antral follicles (LAFs, >4 mm) (Bari *et al*., 2011). The oocytes were retrieved from the different classes of follicles and tested for viability using the trypan blue (0.05% for 2 minutes) staining technique (Gupta *et al*., 2002). Viable oocytes surrounded by a compact multi-layered cumulus with more than three layers of cumulus cells and a homogeneous ooplasm were chosen for further study (Nandi *et al*., 2006).

In-vitro Maturation of Oocytes

Oocytes were washed once with the aspiration medium, consisting of tissue culture medium-199 (TCM-199), 0.3% Bovine serum albumin, gentamicin (50 µg/mL), plus heparin (100 µg/mL). The oocytes were then transferred in groups (5–10/Group) into 50 µL droplets of IVM culture medium in a 35 mm petridish consisting of oocyte maturation medium. The control medium consisting of tissue culture medium-199 (TCM-199), supplemented with 10% fetal bovine serum (FBS), 10 µg/mL of follicle-stimulating hormone (FSH), and 50 µg/mL of gentamicin. Oocytes isolated from LAFs, MAFs and SAFs were exposed with different doses (0-control, 5ng, 10ng, 25 ng, 50 ng, 100 ng, 150 ng) of AREG or NRG-1 or TNF-α. The droplets containing oocytes were then covered with warm (38.5°C) mineral oil, and the petridishes were placed in a CO_2 incubator (38°C, 5 % CO_2 in air, 90-95 % relative humidity) for 24 hours.

Assessment of Oocyte Maturation

Cumulus expansion was assessed at 24 hours of IVM according to the visual assessment of the degree of expansion (cumulus expansion score). Degree-0: no expansion; Degree-1: moderate expansion, cumulus cells were non-homogeneously spread, and clustered cells were still observed, and Degree-2: fully expanded, cumulus cells were homogeneously spread and clustered cells were no longer present (Nandi *et al*., 2006). The parameters of oocytes maturation were the visualization of extrusion of a first polar body in the perivitelline space and expansion of cumulus cell layer (Nandi *et al*., 2002). The oocyte viability was again determined by trypan blue staining technique and was found 90–95%.

Statistical Analysis

Data analysis was done by using the software SPSS-17. Differences between the mean maturation of oocytes were analyzed using one-way analysis of variance (ANOVA), and the respective means were compared using Bonneforni Multiple comparison test (Graph Pad Prism, Graph Pad Software Inc., San Deigo, USA). The percentage values were subjected to arcsine transformation before statistical analysis. Differences between the mean values were considered significant when the P-values were less than 0.05.

Results and Discussion

Effect of AREG on *In-vitro* Maturation of Oocytes Isolated from LAFs, MAFs, and SAFs

In the present study, the maturation rate of oocytes isolated from LAFs in medium containing 50, 100 and 150 ng of AREG was significantly higher than those observed in control and lower concentrations of AREG treated groups (Table 1). This observation of higher oocytes maturation rate in oocytes collected from the LAFs of AREG treated groups of the present study could be because oocytes from large follicles had greater developmental competence than oocytes from SAFs, where it required additional pre-maturation before reaching final maturation due to gonadotrophin surge (Caixeta *et al.,* 2009).

Table 1: Effect of AREG levels on *in vitro* maturation of oocytes isolated from LAFs, MAFs and SAFs

Treatment (AREG; ng/mL)	*LAFs*		*MAFs*		*SAFs*	
	Oocytes n (%)	*Maturation rate n (%)*	*Oocytes (n)*	*Maturation rate n (%)*	*Oocytes n (%)*	*Maturation rate n (%)*
0-Control	57	48 (84.45 ± 1.55)^{a}	57	42 (73.25 ± 1.55)^{a}	50	28 (56.25 ± 1.54)^{a}
AREG (5 ng)	75	62 (83.32 ± 1.89)^{a}	67	49 (73.12 ± 1.89)^{a}	58	33 (57.12 ± 1.29)^{a}
AREG (10 ng)	76	63(84.45 ± 0.94)^{a}	69	51(73.85 ± 0.94)^{a}	67	37(55.85 ± 1.14)^{a}
AREG (25 ng)	59	50 (85.32 ± 1.11)^{a}	62	47 (75.22 ± 1.11)^{a}	56	30 (53.22 ± 1.41)^{a}
AREG (50 ng)	59	53 (89.45 ± 1.67)^{b}	61	44 (72.35 ± 1.67)^{a}	57	33 (57.35 ± 1.27)^{a}
AREG (100 ng)	67	59(88.24 ± 1.95) ^{b}	70	56(80.14 ± 1.95)^{b}	59	37(62.14 ± 1.18)^{b}
AREG (150 ng)	50	44 (88.35 ± 1.78) ^{b}	51	40 (78.95 ± 1.78)^{b}	54	35(64.95 ± 1.32)^{b}

Values with different superscript differ significantly ($p < 0.05$) within column.

Ambekar *et al*. (2015) postulated a strong link between the accumulation of AREG in the large follicles and the proper maturation of oocytes. The maturation of oocytes collected from MAFs was significantly increased in 100 and 150 ng of AREG compared to those observed in control and other groups with AREG. Our results agreed with reports of Richani *et al*. (2014), wherein EGF-peptide/EGFR and cAMP–PKA signaling pathway cross-talk causing intercellular communication in the oocytes were necessary for oocytes developmental competence. It was observed that the maturation of oocytes recovered from SAFs was considerably higher in the groups exposed with 100 and 150 ng of AREG than those observed in control and lower concentrations of AREG-treated groups. This could be due to the beneficial role of AREG on the synthesis of cAMP, oocytes paracrine factors, BMP15 and GDF9 in developing oocytes derived from small antral follicles, which collectively promoted EGFR signaling and enhanced oocytes developmental competence (Sugimura *et al*., 2015). Furthermore, oocytes from SAFs matured at a lower rate than oocytes from MAFs and LAFs, which could be because oocytes from small antral follicles were inherently unresponsive to the full range of EGF family ligands that oocytes acquired EGF responsiveness with advancing folliculogenesis (Ritter *et al*., 2015).

Effect of NRG-I on *In-vitro* Maturation of Oocytes Isolated from LAFs, MAFs, and SAFs

The maturation rate of oocytes isolated from LAFs in medium containing 25, 50, and 100 or 150 ng of NRG-I was significantly higher than those observed in control and lower concentrations of NRG-I treated groups (Table 2). The maturation of oocytes collected from MAFs was significantly increased in groups exposed to 50,100 and 150 ng of NRG-I compared to those observed in control and other lower concentrations groups. This beneficial role of NRG-I might be because NRG-I stimulated oocytes glycolysis and cumulus–oocytes gap-junctional communication, which facilitated the transfer of metabolites from cumulus cells to the oocytes, leading to increased oocytes mitochondrial activity necessary for oocytes developmental competence (Sugimura *et al*., 2014). Further, in the present study, oocytes from MAFs showed significantly lower oocyte maturation than oocytes collected from LAFs and higher than oocytes collected from SAFs. This supported the findings of Lesley *et al.* (2015), wherein oocytes from LAFs and MAFs exhibited more phosporylation of ERK1/2 compared to oocytes from SAFs. The maturation rate of oocytes collected from SAFs revealed no significant difference when compared between control and groups with 5 ng or 10 ng of NRG-I and between 25 ng and 50 ng of NRG-I. However, compared to control and lesser concentrations of NRG-I treated groups, the maturation rate was considerably higher in the group with 150 ng of NRG-I. This might be due to the responsiveness of oocytes from small antral follicles to the EGF-like peptide that improved their developmental competence (Ritter *et al*., 2015).

Effect of TNF-α Levels on *In-vitro* Maturation of Oocytes Isolated from LAFs, MAFs, and SAFs

The maturation rate of oocytes collected from LAFs revealed significantly ($P<0.05$) higher in the group exposed to 25 ng of TNF-α compared to those observed in control and other groups of TNF-α (Table 3). The observation made in this study supported Xiao et al. (2001), wherein TNF-α stimulated the production of protein, which promoted oocyte maturation and ovulation. Further, in the present study, the maturation of oocytes was considerably lower in the groups cultured with higher doses of TNF-α compared to control and groups with 5, 10, and 25 ng of TNF-α. As previously reported by Glister *et al*. (2014), TNF exerted its apoptotic effects through the Type I receptor (TNFR1), whereas other pro-inflammatory impacted growth and differentiation were mediated through the Type II receptor (TNFRII). The observation made in this study coincided with Ma et al. (2006) findings, wherein TNF-α damaged the meiosis spindle structure during porcine oocyte maturation. The maturation rate of oocytes isolated from MAFs was found to be significantly ($P<0.05$) higher in a group consisting of 25 ng of TNF-α compared to those observed in control and other groups with TNF-α. It was reported that TNF-expression in cumulus cells was significantly higher in oocytes with low competence (Assidi *et al*., 2008).

Furthermore, the maturation of oocytes was found to be significantly lower in the higher doses of TNF-α, which

Table 2: Effect of NRG-1 levels on *in vitro* maturation of oocytes isolated from LAFs, MAFs and SAFs

Treatment (NRG-I; ng/mL)	*LAFs*		*MAFs*		*SAFs*	
	Oocytes (n)	*Maturation rate n (%)*	*Oocytes (n)*	*Maturation rate n (%)*	*Oocytes (n)*	*Maturation rate n (%)*
0-Control	64	52 (81.64 ± 1.43)^{a}	65	47 (72.15 ± 0.90)^{a}	44	23 (54.09 ± 1.7)^{a}
NRG-I(5 ng)	52	42 (80.43 ± 1.76)^{a}	53	37 (69.98 ± 1.64^{a}	62	34 (56.46 ± 1.18)^{a}
NRG-I(10 ng)	81	66 (81.81 ± 1.43)^{a}	81	57 (70.64 ± 1.76) ^{a}	65	35 (54.72 ± 0.85)^{a}
NRG-I(25 ng)	64	56 (87.69 ± 1.32)^{b}	64	47 (73.18 ± 1.63)^{a}	55	31 (57.61 ± 1.68)^{b}
NRG-I(50 ng)	59	52 (88.54 ± 1.84)^{b}	62	49 (79.55 ± 1.39)^{b}	57	33 (57.96 ± 1.16)^{b}
NRG-I (100 ng)	66	57 (86.47 ± 1.32)^{b}	64	51 (79.85 ± 1.37)^{b}	54	32 (61.22 ± 1.54) ^{c}
NRG-I (150 ng)	58	51 (87.87 ± 1.87)^{b}	57	44 (77.19 ± 1.38)^{b}	50	33 (66.17 ± 1.59) ^{d}

Values with different superscripts differ significantly ($p < 0.05$) within column.

Table 3: Effect of TNF-α levels on in vitro maturation of oocytes isolated from LAFs, MAFs and SAFs

Treatment	*LAFs*		*MAFs*		*SAFs*	
(TNF-α; ng/mL)	*Oocytes (n)*	*Maturation rate n (%)*	*Oocytes (n)*	*Maturation rate n (%)*	*Oocytes (n)*	*Maturation rate n (%)*
0-Control	64	48 (75.64 ± 1.43)[a]	65	46 (70.15 ± 0.90)[a]	44	22 (50.09 ± 1.7)[a]
TNF-α (5 ng)	66	50 (75.30 ± 1.12)[a]	65	46 (70.19 ± 1.81)[a]	75	37 (50.50 ± 1.02)[a]
TNF-α (10 ng)	78	58 (74.23 ± 0.89)[a]	79	54 (68.29 ± 1.57)[a]	61	29 (49.71 ± 1.72)[a]
TNF-α (25 ng)	56	45 (80.18 ± 1.0)[b]	72	54 (75.79 ± 1.18)[b]	66	29 (45.44 ± 1.24)[b]
TNF-α (50 ng)	67	46 (68.14 ± 1.36)[c]	58	33 (56.21 ± 1.65)[c]	75	24 (37.76 ± 1.72)[c]
TNF-α (100 ng)	71	47 (66.15 ± 1.82)[c]	65	36 (55.65 ± 1.15)[c]	54	21 (39.30 ± 1.27)[c]
TNF-α (150 ng)	63	43 (68.15 ± 1.41)[c]	52	29 (55.19 ± 0.96)[c]	72	27 (38.20 ± 1.57)[c]

Values with different superscripts differ significantly (P<0.05) within column.

could be due to TNF-α impaired microfilament distribution and nucleus status with higher concentrations of TNF-α (Ma *et al*., 2010). When the oocytes were isolated from SAFs, the maturation was significantly lower in 25, 50, 100 and 150 ng of TNF-α when compared to those observed in control and groups with a lower concentration of TNF-α. The observation made in this study agreed with the findings of Ma *et al.* (2010), wherein TNF-α inhibited the maturation rate of oocytes and impaired the quality of oocytes that reached the MII stage with increased concentrations.

Conclusions

It can be concluded that AREG 100 and 150 ng, NRG-1 from 25 to 150 ng, and TNF-α at 25 ng enhanced *in vitro* maturation of ovine oocytes collected from medium and large-sized antral follicles. This knowledge has significant implications for improving the developmental competence of oocytes derived from small follicles.

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References

Ambekar, A.S., Kelkar, D.S., Pinto, S.M., Sharma, R., Hinduja, I., Zaveri, K., Pandey, A., Prasad, T.S., Gowda, H., & Mukherjee, S. (2015). Proteomics of follicular fluid from women with polycystic ovary syndrome suggests molecular defects in follicular development. *The Journal of Clinical Endocrinology & Metabolism, 100*, 744-753.

Assidi, M., Dufort, I., Ali, A., Hamel, M., Algriany, O., Steph Dielemann., &Sirard, A.N.M. (2008). Identification of potential markers of oocytes competence expressed in bovine cumulus cells matured with follicle stimulating hormone and/or phorbol myristate. *Biology of Reproduction*, *79,* 209-222.

Bari, M.A., Kabir, M.E., Sarker, M.B., Ahna khan., &Moniruzzaman, M. (2011). Morphometric analysis of ovarian follicles of Black Bengal goats during winter and summer season. *Bangladesh Journal of Animal Sciences, 40* (1&2), 51-55.

Caixeta, E.S., Ripamonte, P., Franco, M.M., Junior, J.B., &Dode, M.A. (2009). Effect of follicle size on mRNA expression in cumulus cells and oocytes of Bos indicus: an approach to identify marker genes for developmental competence. *Reproduction, Fertility and Development*, *21*, 655-664.

Conti, M., Hsieh, M., Zamah, A.M., & Oh, J.S. (2012). Novel signaling mechanisms in the ovary during oocytes maturation and ovulation. Molecular and *Cellular Endocrinology*, *356*(1-2), 65-73

Gall, L., Chene, N., Dahirel, M., Ruffini, S., & Boulesteix, C. (2004). Expression of epidermal growth factor receptor in the goat cumulus-oocytes complex. *Molecular Reproduction and Development*, *67*, 439-445.

Glister, C., Hatzirodos, N., Hummitzsch, K., Knight, P.G., & Rodgers, R.J. (2014). The global effect of follicle-stimulating hormone and tumour necrosis factor α on gene expression in cultured bovine ovarian granulosa cells. *BMC Genomics*, *15*, 72-82.

Graph Pad Prism, Version 4.00 for Windows. (2004). GraphPad Software Inc. San Deigo, California, USA.

Gupta, P. S. P., Nandi, S., Ravindranatha, B. M., &Sarma, P. V. (2002). Trypan blue staining to differentiate live and dead buffalo oocytes and its effect on embryo development *in vitro*. *Buffalo Journal*, *18*, 321-329.

Lesley, J., Ritter., Sugimura, S., Robert, B., & Gilchrist. (2015). Oocytes induction of EGF responsiveness in somatic cells is associated with the acquisition of porcine oocytes developmental competence. *Endocrinology*, *156*(6), 2299-2312.

Ma, C.H., Qiao, J., Chen, G.A., Zhang, Y.L.,& Yang, C.S. (2006). The significance offollicular fluid TNF–a in endometriosis associated infertility. *Chinese Journal of Clinical Obstetrics and Gynecology*, *7*, 333-335.

Ma, C.H., Yan, L.Y., Qiao, J., Sha, W., Li, B.L., Chen, Y.,& Sun, Q.Y. (2010). Effects of tumor necrosis factor–alpha on porcine oocytes meiosis progression, spindle organization, and chromosome alignment. *Fertility and Sterility*, *93*(3), 920–926.

Nandi, S., Ravidranath, B.M., Gupta, P.S.P.,&Sarma, P.V. (2002). Timing of sequential changes in cumulus cells and first polar body extrusion during *in vitro* maturation of buffalo oocytes. *Theriogenology*, *57*, 1151-1159.

Nandi, S., Girishkumar, V., Gupta, P.S.P., Ramesh., H.S.,&Manjunatha, B.M. (2006). Effect of ovine follicular fluid peptide on follicle, oocytes, and somatic cell culture in buffalo (bubalusbubalis). *Animal Reproduction*, *3*, 61-69.

Noma, N., Kawashima, I., Fan, H.Y., Fujita, Y., Kawai, T. &Tomoda, Y., Mihara, T., Richards, J.S. & Shimada, M. (2011). LH-Induced Neuregulin 1 (NRG1) type III transcripts control granulosa

cell differentiation and oocyte maturation. Molecular Endocrinology, *25*(1), 104-116.

Richani, D., Ritter, L.J, Thompson, J.G.,& Gilchrist, R.B. (2013). Mode of oocyte maturation affects EGF-like peptide function and oocyte competence. Molecular human reproduction, *19*, 500-509.

Richani, D., Sutton–mcdowall, M.L., Frank, L.A., Gilchrist, R.B., &Thompson, J.G. (2014). Effect of epidermal growth factor-like peptides on the metabolism of in vitro– matured mouseoocytes and cumulus cells. *Biology of Reproduction*, *90*, 49-56.

Ritter, L.J., Sugimura, S., & Gilchrist, R.B. (2015). Oocytes induction of EGF responsiveness in somatic cells is associated with the acquisition of porcine oocytes developmental competence. *Endocrinology*, *156*, 2299-2312.

Soares, A.C.S., Sanches, L., Barros, R.G., Lima, P.F., Lodde, V., Luciano, A.M., Price, C., &Buratini. J. (2015). Effects of follicle stimulating hormone (FSH) and amphiregulin (AREG) on meiosis dynamics of oocytes undergoing *in vitro* maturation. *Animal Reproduction*, *12*(3), 586-590.

Sugimura, S., Ritter, L.J., Sutton-mcdowall, M.L., Mottershead, D.G., Thompson, J.G.,& Gilchrist, R.B. (2014). Amphiregulin cooperates with bone morphogenetic protein 15 to increase bovine oocytes developmental competence: effects on gap junction mediated metabolite supply. *Molecular Human Reproduction*, *20*, 499-513.

Sugimura, S., Ritter, L.J., Rose, R., Thompson, J.G., Smitz, J., Mottershead, D.G., & Gilchrist, R.B. (2015). Promotion of EGF receptor signaling improves the quality of low developmental competence oocytes. *Developmental Biology*, *403*(2), 139-149.

Xiao, P., Tekpetey, F.R., & Armstrong, D.T. (2001). Effect of IGF-I on pig oocytes maturation, fertilization, and early embryonic development in vitro, and on granulosa and cumulus cell biosynthetic activity. *Molecular Reproduction and Development*, *38***,** 373-379.

Yarden, Y., &Sliwkowski, M.X. (2001). Untangling the ErbBsignalling network. *Nature Reviews Molecular Cell Biology*, *2*, 127-113.